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# Table of Contents

	Page number
Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	10
Appendices.....	12

**Introduction:**

The main goal of this project is to study the anti-tumor activity of p202 and its application to gene therapy in breast cancer model systems. P202, an interferon (IFN)-inducible protein, interacts with several important regulatory proteins which participate signaling transduction pathways those are involved in cell cycle, cell differentiation and growth. These protein-protein interactions primarily lead to transcription repression of target genes, causing cellular growth arrest or differentiation (ref. 1-10). Our previous studies have shown that p202 could suppress both *in vitro* and *in vivo* cell growth and tumorigenicity of human breast cancer cells (ref. 11). We also found that p202 can directly interact with NF- $\kappa$ B and inhibit TNF- $\alpha$  induced NF- $\kappa$ B activation. The p202-mediated inactivation of NF- $\kappa$ B sensitizes breast cancer cells to TNF- $\alpha$  induced apoptosis (ref. 12). The major objectives of this grant application are to determine the effects of p202 on the sensitivity of breast cancer cells to anticancer agents; to test the anti-tumor activity of p202 in breast cancer cells using pre-clinical gene therapy strategies in an orthotopic breast cancer animal model; and to evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotopic breast cancer animal model. The success of the pre-clinical experiments in this grant application could lead to the translation of the laboratory research to clinical products and thus benefit breast cancer patients.

## **Body:**

### **A. Specific Aims: (no changes)**

**Specific Aim 1:** To determine the effect of p202 on the sensitivity of breast cancer cells to anti-cancer agents.

**Specific Aim 2:** To test the anti-tumor activity of p202 in breast cancer cells using pre-clinical gene therapy strategies in an orthotopic breast cancer animal model.

**Specific Aim 3:** To evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotopic breast cancer animal model.

### **B. Studies and Results:**

In the last two years, we have developed a p202-mediated delivery system for therapeutic efficacy study in animal models. We further investigated the biological functions of p202. Our results have demonstrated that adenovirus-mediated p202(Ad-p202) gene transfer and expression indeed could cause potent growth-suppression and sensitization to TNF- $\alpha$  induced apoptosis in breast cancer cells. In addition, p202 overexpression alone induces apoptosis and that may contribute to the p202-mediated multiple anti-tumor activity. Our data suggested that the activation of caspase (in particular, caspase-3) may be critical for Ad-p202 to exert full apoptotic effect in breast cancer cells. Importantly, Ad-p202 treatments resulted in significant tumor suppression in breast cancer xenograft model. A manuscript entitled "Pro-apoptotic and anti-tumor activities of adenovirus-mediated p202 gene transfer" by Ding, Y. et al. submitted to *Clinical Cancer Research* is currently in revision.

The progress of each specific aim is discussed below:

**Specific Aim 1:** To determine the effect of p202 on the sensitivity of breast cancer cells to anti-cancer agents.

To study the biological effect of p202 *in vitro*, we have constructed adenoviral vector-p202 delivery system to infect breast cancer cells, MDA-MB-468. Our studies have shown that: 1) Ad-p202 delivery system is feasible and efficient (please see Appendix Figure 1); 2) overexpression of p202 inhibits growth of breast cancer cell (Figure 2); 3) Ad-p202 infected breast cancer cells are sensitized to TNF- $\alpha$ -induced apoptosis (Figure 3); and 4) Ad-p202 infected breast cancer cells are sensitized not only to  $\gamma$ -irradiation-induced apoptosis but also to anti-cancer drugs, such as Taxol and CDDP, -induced apoptosis (Figure 6). To examine the molecular mechanism of p202's pro-apoptotic activity, we have treated Ad-p202 infected breast cancer cells with a pan caspase inhibitor, Z-VAD. We have found that activation of caspase is required for Ad-p202-mediated apoptosis in infected breast cancer cells. To further support our observation, we have infected MCF-7 cells, which are caspase-3 null breast cancer cells, with Ad-p202, the infected MCF-7 cells are unable to induce apoptosis. In addition, we treated Ad-p202 infected MDA-MB-468 cells with a caspase-3 specific inhibitor, Z-DEVD-fmk, the treated cells failed to go into apoptosis (Figure 4). Our results indicated that the caspase-3 is critical for Ad-p202-mediated apoptosis.

**Specific Aim 2:** To test the anti-tumor activity of p202 in breast cancer cells using pre-clinical gene therapy strategies in an orthotopic breast cancer animal model.

To test the efficacy of Ad-p202 *in vivo*, we have performed *in vivo* tumorigenesis on female nude mice. The tumor-bearing mice then were subjected to intra-tumor injection or to systemic gene therapy via tail vein injection with Ad-p202 (or liposome delivery system, CMV-p202/SN2.) Our studies have demonstrated that p202 exhibits anti-tumor activity in intra-tumor and in tail vein injection (Figures 5 & 7). To further understand the anti-tumor mechanism of p202 *in vivo*, the treated tumor section was studied by TUNEL assay and immunohistochemical analysis. Our results have suggested that the anti-tumor activity of p202 *in vivo* is correlated with tumor cell death and downregulation of an angiogenic factor, VEGF (Figure 8).

**Specific Aim 3:** To evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotopic breast cancer animal model.

In the past two years, we have successfully established Ad-p202 and CMV-p202/SN2 delivery systems to test its pro-apoptotic and anti-tumor activities *in vitro* and *in vivo*.

We will evaluate the therapeutic efficacy in combination with anti-cancer agents, such as Taxol, in an orthotopic breast cancer animal model. This Aim will be an important area of focus in the subsequent years of research within this grant supported period.

### **Key Research Accomplishments:**

- Ad-p202 infection inhibits cell proliferation in MDA-MB-468 breast cancer cells.
- Ad-p202 infection induces apoptosis and sensitizes MDA-MB-468 cells to apoptosis by TNF- $\alpha$
- P202 expression enhances apoptosis induced by  $\gamma$ -irradiation, taxol, or CDDP.
- Activation of caspase-3 is critical for Ad-p202-mediated apoptosis in breast cancer cells, MDA-MB-468.
- Anti-tumor effect by systemic delivery of Ad-p202 on an orthotopic breast cancer xenograft model in nude mice.
- *In vivo* anti-tumor effect by a systemic delivery of Ad-p202 and CMV-p202/SN2.
- Immunohistochemical analysis of p202-treated tumors which are from mice bearing breast tumors derived from MDA-MB-468.

### **Reportable Outcomes:**

Ding, Y., Wen, Y., Spohn, B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., **Hung, M.-C.**, and Yan, D.-Y. Pro-apoptotic and anti-tumor activities of adenovirus-mediated p202 gene transfer. *Clinical Cancer Research*, 2002, in revision.

### **Conclusions:**

Based upon our studies, p202, an interferon-inducible protein, has pro-apoptotic and anti-tumor activities *in vitro* and *in vivo*, respectively. Its pro-apoptotic activity may require activation of caspase-3 pathway; and its anti-tumor activity may be related to induce



apoptosis and inhibit angiogenesis in tumor cells. P202 also sensitizes breast cancer cells to TNF- $\alpha$ ,  $\gamma$ -irradiation, Taxol and CDDP-induced apoptosis *in vitro*. It is worth noting that in another our observation, the p202 suppresses metastasis and angiogenesis in a human pancreatic cancer model system (ref. 13). Taken together, p202 could be a potentially therapeutic gene against breast cancer. Therefore, we will continue to investigate whether the combination of p202 and anti-cancer drugs or TNF- $\alpha$  or  $\gamma$ -irradiation therapy might achieve synergistic (or additive) therapeutic efficacy against breast cancer *in vivo*. The success of the pre-clinical experiments in this proposal would fulfill the purpose of directing the basic science research in laboratory to clinical products to benefit breast cancer patients.

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**Appendices:**

**Attached manuscript** by Ding, Y., Wen, Y., Spohn, B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., **Hung, M.-C** and Yan, D.-Y. Pro-apoptotic and anti-tumor activities of adenovirus-mediated p202 gene transfer. *Clinical Cancer Research*, 2002, in revision.

## **Pro-apoptotic and Anti-tumor Activities of Adenovirus-Mediated p202 Gene Transfer<sup>1</sup>**

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Running Title: Pro-apoptotic and anti-tumor activities of Ad-p202 gene transfer

Key words: adenovirus, apoptosis, caspases, TNF- $\alpha$

## ABSTRACT

*Purpose and Experimental Design:* p202, an interferon-inducible protein, is a member of the murine 200-amino acid repeat family. Enforced p202 expression in stable cancer cell lines resulted in growth inhibition *in vitro* and tumor suppression *in vivo*. However, to study the immediate effect of p202 and to test the potential efficacy of p202 treatment, an efficient gene delivery system for p202 is required. For these purposes, an adenoviral vector expressing the p202 gene (Ad-p202) was generated. We examined the effects of Ad-p202 infection on human breast cancer cells. Furthermore, we tested the efficacy of Ad-p202 treatment on breast and pancreatic cancer xenograft models.

*Results:* We found that Ad-p202 infection induces growth inhibition and sensitizes the otherwise resistant cells to TNF- $\alpha$ -induced apoptosis. In addition, we demonstrated for the first time that Ad-p202 infection induces apoptosis, and that requires the activation of caspase-3 for full apoptotic effect. More importantly, we showed the efficacy of Ad-p202 treatment on both breast and pancreatic cancer xenograft models, and this anti-tumor effect correlated well with enhanced apoptosis in Ad-p202-treated tumors.

*Conclusions:* We conclude that Ad-p202 is a potent growth inhibitory, pro-apoptotic and tumor-suppressing agent. Ad-p202 may be further developed into an efficient therapeutic agent for human cancer gene therapy.

## INTRODUCTION

p202, an IFN-inducible, chromatin-associated protein, belongs to a murine 200-amino acid repeat family (1, 2). The unique feature of p202 is illustrated by its ability to interact with several important transcriptional regulators that include E2Fs, Rb and the pocket

proteins, p130 and p107, Fos/Jun, c-Myc, NF- $\kappa$ B, and p53BP-1 (reviewed in (3)), resulting in transcriptional repression of genes that are up-regulated by these transcriptional regulators. The exact role of p202 in the IFN-mediated signal pathway is not well defined. However, consistent with the multiple anti-tumor activities of IFN (4), enforced expression of p202 in stable murine fibroblasts and human cancer cell lines leads to retardation of cell growth and suppression of transformation phenotype (5-8). Furthermore, p202 stably transfected breast cancer cells are sensitized to TNF- $\alpha$ -induced apoptosis (8), and that effect is associated with inactivation of the TNF- $\alpha$ -induced NF- $\kappa$ B via p202/NF- $\kappa$ B interaction. We postulated that p202 sensitizes cancer cells to TNF- $\alpha$ -induced apoptosis by inactivating NF- $\kappa$ B that, in turn, turns off NF- $\kappa$ B-activated anti-apoptotic gene expression leading to enhanced TNF- $\alpha$ -induced cell killing (8).

To generate a p202-based therapeutic agent for efficacy study in animal models and a tool to study the biological function of p202, we constructed Ad-p202. In this study, we showed that Ad-p202 infection into breast cancer cells resulted in growth inhibition and sensitization to TNF- $\alpha$ -induced apoptosis. Interestingly, we found that Ad-p202 infection alone induces apoptosis in breast cancer cells and the activation of caspase-3 is critical for this process. More importantly, we demonstrated the efficacy of Ad-p202 treatment in human breast and pancreatic cancer xenograft models through either intra-tumor (i.t.) or intra-venous (i.v.) injection. This anti-tumor activity correlated well with p202 expression and apoptosis in Ad-p202-treated tumors. Together, our results suggest that Ad-p202 is a potent growth inhibitory, pro-apoptotic, and anti-tumor agent that could



be further developed to become an effective therapeutic agent for cancer gene therapy treatment.

## **MATERIALS AND METHODS**

**Generation of Ad-p202.** Ad-p202 was constructed according to the protocol described previously (9). p202 cDNA (6) was subcloned into an adenovirus vector (pAdTrack-CMV) that carries a CMV promoter-driven green fluorescence protein (GFP). A separate CMV promoter directs p202 cDNA. A control virus, an adenoviral vector expressing luciferase gene (Ad-Luc) and GFP was likewise generated. The expression of GFP gene enabled us to monitor the infection efficiency by direct observation using a fluorescence microscope.

***In Vitro* Growth Assays.** MDA-MB-468 human breast cancer cells were maintained in DMEM/F-12 medium (HyClone Laboratories, Inc.) supplemented with 10% (v/v) fetal bovine serum. For MTT assay,  $2 \times 10^3$  cells were plated in 96-well culture plates in 0.1 ml of culture medium. Ad-p202 or Ad-Luc was added at multiplicity of infection (MOI) of 200 on the next day. At different times indicated, 20  $\mu$ l of MTT (5 mg/ml stock solution) were added to each well. Cells were cultured for an additional 2 hours, then 100  $\mu$ l of lysis buffer (20% SDS in 50% *N, N*-dimethylformamide, pH 4.7) were added to each well followed by five hours of incubation, and then absorbance was measured at 570 nm. [ $^3$ H] Thymidine incorporation assay was done as described previously (10).

**Apoptosis Assays.** Flow cytometry analysis: cells were collected at 72-h post-infection, washed once with phosphate-buffered saline (PBS) and suspended in 0.5 ml of PBS containing 0.1% (v/v) Triton X-100 for nuclei preparation. The suspension was filtered through a nylon mesh, then adjusted to a final concentration of 0.1% (w/v) RNase and 50 µg/ml propidium iodide. Apoptotic cells were quantitated by FACScan cytometer. DNA Fragmentation Assay was carried out as described previously (10).

**Western Blot Analysis.** MDA-MB-468 cells treated with or without TNF- $\alpha$  (R & D Systems, Inc., Minneapolis, MN) were infected with Ad-p202 or Ad-Luc at MOI of 200. 72-h post-infection, cells were lysed with RIPA lysis buffer. The protein extracts were subject to SDS-PAGE followed by western blotting according to the procedure described previously (8). Goat anti-p202 polyclonal antibody and anti-PARP antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Transduction Labs (Lexington, KY), respectively. Caspase inhibitors, Z-VAD and Z-DEVD-fmk were purchased from Enzyme Systems Products (Livermore, CA).

**Gel-Shift Assay.** The NF- $\kappa$ B gel-shift assay was performed as described previously (10).

**Ad-p202 gene therapy in human cancer xenograft models.** For orthotopic breast cancer xenograft model: MDA-MB-468 cells ( $2 \times 10^6$  cells) were implanted in mammary fat pads (2 tumors per mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups: group 1, Ad-Luc; and group 2, Ad-p202. For intra-tumor

injection,  $1 \times 10^9$  pfu of viruses per treatment were administered. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for seven weeks and once a week thereafter. For tail vein injection,  $5 \times 10^8$  pfu of viruses per treatment were administered. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for five weeks and once a week thereafter. For pancreatic cancer xenograft model: Panc-1 cells ( $1 \times 10^6$  cells) were subcutaneously implanted into two flanks of each nude mouse. Tumor-bearing mice were divided into two treatment groups: group 1, Ad-Luc (5 mice/10 tumors), and group 2, Ad-p202 (5 mice/10 tumors), at  $5 \times 10^8$  pfu via intra-tumor injection. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week throughout.

**Immunohistochemical analysis of p202 expression and apoptosis.** Mice were sacrificed 24 h after the last treatment. Tumors obtained from Ad-p202 or Ad-Luc treated mice bearing either breast or pancreatic tumors were then excised and fixed with formalin and embedded in paraffin. Immunohistochemical analysis of p202 protein expression was performed according to the protocol described previously (11). Tumor sections were incubated with goat polyclonal antibody specific for p202 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with biotinylated rabbit anti-goat IgG, and subsequently with avidin-biotin-peroxidase before visualization. TUNEL {TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labeling} assay was performed to detect the ends of degraded DNA fragments induced by apoptosis according to the protocol described previously (12).

## RESULTS

**Ad-p202 mediates p202 expression in breast cancer cells.** To test the efficiency and to monitor the expression of p202 protein by Ad-p202 infection, we infected MDA-MB-468 breast cancer cells with either Ad-p202 or Ad-Luc followed by fluorescence microscopy and western blot analysis, respectively. As shown in Fig. 1A, Ad-p202 and Ad-Luc infection at MOI of 200 exhibited more than 90% infection efficiency as indicated by the GFP-positive cells shown in a representative field (Fig. 1A, right panels). Same cells are shown in phase contrast images (Fig. 1A, left panels). The mock-infected cells (control) showed no GFP expression. In addition to MDA-MB-468, we found that Ad-p202 could infect a panel of other human breast cancer cell lines (e.g., MDA-MB-453, MDA-MB-435, MDA-MB-231, and MCF-7), albeit, with various infection efficiency rates (data not shown). We chose the MDA-MB-468 cell line for subsequent studies because it is tumorigenic in mouse xenograft model and has relatively high infection efficiency by Ad-p202. The expression of p202 protein in Ad-p202 infected cells was further analyzed by western blot using p202-specific antibody. Fig. 1B shows that, while the mock- and Ad-Luc-infected cells have no p202 expression, Ad-p202 infection efficiently directed p202 expression in MDA-MB-468 cells in a dose-dependent manner. These results clearly demonstrate that Ad-p202 infection adequately directs p202 expression in MDA-MB-468 cells.

**Ad-p202 infection reduces breast cancer cell growth.** To assess the effect of Ad-p202 infection upon cell growth, we infected MDA-MB-468 cells with either Ad-p202 or Ad-Luc followed by *in vitro* growth assays such as MTT and <sup>3</sup>H-thymidine incorporation.

As shown in Fig. 2, while the mock infection (control) and Ad-Luc infection have no growth inhibitory effect on MDA-MB-468 cells, Ad-202 infection significantly hampered cell growth (Fig. 2A) and DNA synthesis rates (Fig. 2B). This observation strongly indicates that Ad-p202 infection inhibits cell growth in breast cancer cells, and that is congruent with our previous findings using stable cancer cell lines (7, 8).

**Ad-p202 infection induces apoptosis in breast cancer cells.** Without stress signals, the p202-stable cancer cell lines do not exhibit apoptotic phenotype (7, 8). It is possible that p202 stable cell lines isolated after a vigorous selection process may possess a physiologically tolerant level of p202. The fact that only a small number of p202 stable cell lines were obtained by colony-forming assay (7, 8) raises the possibility that p202 expression alone may induce apoptosis. To test that possibility, we infected MDA-MB-468 cells with Ad-p202 or Ad-Luc followed by flow cytometry analysis to detect apoptosis by measuring the cell population in sub-G1 phase of cell cycle. As shown in Fig. 3A, although Ad-Luc infection induced a modest apoptosis, (compared lane 1 with lane 3), Ad-p202 infection (lane 5) caused significantly more apoptosis (>20%) than that of the mock (lane 1) and Ad-Luc (lane 3) infection. That observation was further confirmed by two other apoptosis assays: first, poly (ADP-ribose) polymerase (PARP) cleavage assay in which the full-length PARP (116 kD) is cleaved by caspases into a fragment of approximately 85 kD (Fig. 3B); and second, a DNA fragmentation assay that is based on the activated endonucleases during apoptosis (Fig. 3C). Ad-p202 infection resulted in a marked increase of PARP cleavage product (85 kD) (Fig. 3B, lane 5 and Fig. 4A, lane 3) and an enhancement of DNA fragmentation (Fig. 3C, lane 5). In contrast,

Ad-Luc infection yielded a minimum amount of 85 kD PARP cleavage product (Fig. 3B, lane 3 and Fig. 4A, lane 2) as well as a near basal level of DNA fragmentation (Fig. 3C, compare lane 1 and lane 3). Together, our results strongly indicate that Ad-p202 alone induces apoptosis in MDA-MB-468 cells. Given that the MDA-MB-468 cell line harbors mutant p53 (13), Ad-p202-mediated apoptosis thus appears to be independent of p53 status.

**Caspase-3 activation is critical for p202-mediated apoptosis.** Since caspases are activated during apoptosis and have a variety of substrates including PARP (14), the cleavage of PARP in Ad-p202-infected cells suggests that the activation of caspase may be involved in Ad-p202-induced apoptosis. To test that hypothesis, we infected MDA-MB-468 cells with Ad-p202 or Ad-Luc in the presence or absence of a pan caspase inhibitor, Z-VAD. The intensity of PARP full-length and cleavage product bands on western blot were measured using NIH Image 1.62 software. The percentage of 85 kD product was calculated by setting the total intensity of both 116 kD and 85 kD bands in each lane at 100%. As shown in Fig. 4A, the addition of Z-VAD attenuates Ad-p202-induced apoptosis as indicated by the reduced (though not completely eliminated) level of PARP cleavage 85 kD product from 57.4% (lane 3) to 13.3% (lane 5), whereas Z-VAD has no effect on PARP cleavage in Ad-Luc-infected cells (lane 4). This result supports the idea that the activation of caspases is at least in part required for Ad-p202 to induce full apoptotic effect. Since PARP is also a substrate for caspase-3 that is considered to be a crucial enzyme commonly activated during apoptosis (14), we examined if the activation of caspase-3 plays a role in Ad-p202-induced apoptosis. To that end, we

infected MDA-MB-468 cells with either Ad-p202 or Ad-Luc in the presence of a caspase-3 specific inhibitor, Z-DEVD-fmk (15). As shown in Fig.4A, the level of PARP cleavage product in Ad-p202-infected MDA-MB-468 cells is significantly reduced to 8.9% (lane 7) as compared with that without Z-DEVD-fmk (57.4%, lane 3). As a control, no detectable PARP cleavage was observed in Ad-Luc-infected cells treated with Z-DEVD-fmk (lane 6). Thus, this result suggests that the activation of caspase-3 is critical for Ad-p202-mediated apoptosis. To further confirm that observation, we infected Ad-p202 or Ad-Luc into a caspase-3-null breast cancer cell line, MCF-7 (16) followed by flow cytometry analysis. We observed that, though with greater than 90% infection efficiency at MOI of 2000 based on GFP fluorescence microscopy (data not shown), Ad-p202 infection yielded near background level of apoptosis as that of the controls, i.e., mock and Ad-Luc infection (Fig. 4B). The inability of Ad-p202 to induce apoptosis in MCF-7 cells is not due to the lack of p202 expression since p202 protein was readily expressed as determined by western blot (Fig. 4B). Therefore, our data suggest that the activation of caspases, and caspase-3 in particular, is critical for Ad-p202 to exert full apoptotic effect.

**Ad-p202 infection sensitizes breast cancer cells to TNF- $\alpha$ -induced apoptosis.** We tested if Ad-p202 infection could also sensitize breast cancer cells to TNF- $\alpha$ -induced apoptosis (8). Although MDA-MB-468 cells appear to be resistant to TNF- $\alpha$  (50 ng/ml)-induced apoptosis (Fig. 3A, lanes 1 and 2; Fig. 3B, lanes 1 and 2; and Fig. 3C, lanes 1 and 2), the combination of TNF- $\alpha$  and Ad-p202 induced a massive cell killing (Fig. 3A, lane 6; Fig. 3B, lane 6; and Fig. 3C, lane 6). Based on flow cytometry analysis data (Fig. 3A),

we determined if the observed apoptotic effect of TNF- $\alpha$  and Ad-p202 combined treatment (lane 6) is synergistic using a formula  $C < A \times B\% \times 70\%$  (17) (in which C is % of survival cells after combined treatment, and A and B are % of survival cells after each treatment). In this case, C is 49.9% (Fig. 3A, lane 6), A is TNF- $\alpha$  treatment alone (95.5%, Fig. 3A, lane 2), and B is Ad-p202 infection alone (75%, Fig. 3A, lane 5). The result,  $49.9\% < 50.1\%$  ( $= 95.5\% \times 75\% \times 70\%$ ), suggests that the apoptotic effect of Ad-p202 and TNF- $\alpha$  in combination is indeed synergistic. In addition, the combined treatment of TNF- $\alpha$  and Ad-Luc infection did not cause a synergistic killing as compared with that of either treatment alone (Fig. 3A, lanes 2, 3, and 4; Fig. 3B, lanes 2, 3, and 4; Fig. 3C, lanes 2, 3, and 4), suggesting that such sensitization to TNF- $\alpha$ -induced apoptosis is specific to p202 expression. Since p202-mediated sensitization to TNF- $\alpha$ -induced apoptosis correlated with the inactivation of NF- $\kappa$ B, specifically, via the loss of NF- $\kappa$ B DNA binding activity (8), we then tested whether Ad-p202 infection affects TNF- $\alpha$ -induced NF- $\kappa$ B DNA binding activity. As shown in Fig 3D, we observed a complete abolishment of TNF- $\alpha$ -induced NF- $\kappa$ B DNA binding activity in Ad-p202-infected MDA-MB-468 cells (Fig. 3D, compare lanes 1, 3, 5 and 6). As controls, TNF- $\alpha$ -induced NF- $\kappa$ B DNA binding activity (lane 6) can be readily competed by cold wild type NF- $\kappa$ B DNA binding site (lane 7) but to a less extent by cold mutant probe (lane 8). Ad-Luc infection also reduces the TNF- $\alpha$ -induced NF- $\kappa$ B DNA binding activity somewhat but to a less extent than Ad-p202 (lanes 4 and 5). Together, our data suggest that Ad-p202 infection could sensitize otherwise resistant MDA-MB-468 cells to apoptosis induced by TNF- $\alpha$ , and that correlates with a loss of TNF- $\alpha$ -induced NF- $\kappa$ B DNA binding activity.



**Anti-tumor activity of Ad-p202 in cancer xenograft models:** To test the efficacy of Ad-p202 treatment in an orthotopic breast cancer xenograft model, we implanted MDA-MB-468 cells ( $2 \times 10^6$  cells) into mammary fat pads of female nude mice. Treatment began when tumor size reached  $\sim 0.5$  cm in diameter (about 2 weeks after implantation). We then treated tumor-bearing mice (5 mice/10 tumors per treatment group) with either Ad-p202 or the control virus, Ad-Luc, ( $1 \times 10^9$  pfu per treatment) via intra-tumor injection. Treatments were administered twice per week for seven weeks and once a week thereafter. Tumor size was measured by using the formula [ $= 1/2 \times L \times S^2$  (long (L) and short (S) diameters measured)]. As shown in Fig. 5A, Ad-p202-treated mice had much reduced tumor growth as compared with that treated with Ad-Luc. Since breast cancer is a metastatic disease, it is critical to develop a systemic delivery system for p202 gene transfer. Although the anti-tumor effect by intra-tumor treatment is encouraging, no report has shown a therapeutic effect by systemic administration of p202 in cancer xenograft model. We then performed systemic gene therapy experiments by treating tumor-bearing mice with Ad-p202 or Ad-Luc ( $5 \times 10^8$  pfu per treatment) through tail vein injection. Treatments were administered twice per week for five weeks and once a week thereafter. As shown in Fig. 5B, Ad-p202-treated mice (3 mice/6 tumors) had significantly reduced tumor growth rate as compared with the control, i.e., Ad-Luc-treated mice (2 mice/4 tumors). This observation strongly suggests the feasibility of a systemic p202-based gene therapy treatment for breast cancer. Since Ad-p202 induces apoptosis *in vitro* (Fig. 3), it is likely that the observed anti-tumor activity may correlate with enhanced apoptosis in Ad-p202-treated tumors. To test this possibility, we examined the presence of apoptosis in breast tumors treated with Ad-p202. As shown in

Fig. 5C, p202 expression was readily detected by immunohistochemical staining in Ad-p202-treated tumors but not in tumors treated with Ad-Luc. Interestingly, strong p202 expression was found in endothelial cells of a tumor blood vessel. It may be due to systemic delivery of Ad-p202. As predicted, apoptosis, determined by TUNEL assay, is prevalent in Ad-p202-treated tumors but not in Ad-Luc-treated tumors (Fig. 5C). The arrows indicate the representatives of apoptotic cells. This observation is consistent to our *in vitro* data that showed p202 expression induces apoptosis (Fig. 3).

To further confirm the anti-tumor activity of Ad-p202 in other cancer cells, we tested the efficacy of Ad-p202 treatment in a human pancreatic cancer xenograft model (18). Briefly, Panc-1 cells were subcutaneously implanted into two flanks of each nude mouse. Each treatment group (Ad-p202 or Ad-Luc) consisted of five mice with a total of 10 tumors. Twice a week, intra-tumor injection ( $5 \times 10^8$  pfu per treatment) began when tumor size reached ~ 0.3 cm in diameter. As shown in Fig. 5D, Ad-p202 treatment significantly reduced tumor growth as compared with that of Ad-Luc treatment. Again, p202 protein expression correlates well with increased apoptosis in Ad-p202-treated tumors, but not in that treated by Ad-Luc (Fig. 5E). Taken together, the above observations strongly indicate that p202 is a potent tumor suppressing agent by which apoptosis contributes to the multiple p202-mediated anti-tumor activities.

## DISCUSSION

In this report, we showed, like p202 stable breast cancer cell lines (8), Ad-p202 infection in MDA-MB-468 breast cancer cells resulted in growth inhibition and sensitization to

TNF- $\alpha$ -induced apoptosis. Importantly, we demonstrated for the first time that Ad-p202 infection alone induces apoptosis *in vitro*. The correlation between p202 expression and enhanced apoptosis observed in Ad-p202-treated tumors also supports the *in vitro* observation. However, it is possible that the apoptosis could be the result of an artifact caused by co-expression of p202 and adenoviral proteins. We ruled out that possibility because infection of Ad-Luc into a p202 stable cell line, 453-p202 (8), did not result in enhanced apoptosis as compared with that of the vector control cells infected by Ad-Luc (data not shown). This result thus strongly suggests that the Ad-p202-induced apoptosis is not likely due to cooperation between p202 and certain adenoviral proteins during infection. Rather, it indicates that certain cellular apoptotic pathway was activated by p202 expression. Indeed, as shown in Fig. 4, the Ad-p202-induced apoptosis requires caspase-3 activation to achieve a full apoptotic effect.

Here, we demonstrated the feasibility of using Ad-p202 in pre-clinical gene therapy settings. In particular, Ad-p202 treatment by i.t. or i.v. injection resulted in significant tumor suppression in an orthotopic breast cancer xenograft model. Our data are consistent to that reported previously using p202 delivery systems other than adenoviral vector, i.e., polymer and liposome (8, 18). The efficacy of systemic Ad-p202 treatment is encouraging because it shows that Ad-p202 had overcome immunological (nude mice possess immune response, albeit, much reduced), physiological, and structural barriers inside and outside the blood vessels to reach tumor cells and unloads the p202 therapeutic gene (19). This result is the first demonstration of efficacy by systemic treatment of p202. Although toxicity, if any, associated with Ad-p202 treatment remains to be

determined, our results nevertheless raise the possibility of using p202-based gene therapy in systemic cancer treatment. In Ad-p202-treated tumors, we also found reduced level of an angiogenic marker, VEGF, (data not shown). This observation is consistent to the ability of p202 to inhibit angiogenesis (18).

In addition to prostate (data not shown) and breast cancer xenograft models (this study and (8)), the fact that Ad-p202 treatment resulted in anti-tumor effect on a pancreatic cancer xenograft model (this study and (18)), suggesting a general application of p202-based gene therapy in cancer treatment. In addition, since p202 sensitizes cells to TNF- $\alpha$ -induced apoptosis (this study and (8)), our data further support the possible use of Ad-p202/TNF- $\alpha$  combined therapy to achieve better efficacy especially for cancer cells that are resistant to TNF- $\alpha$  therapy. Experiments are underway to test this possibility in animal models. Together, our data presented here strongly suggest Ad-p202 is a potent therapeutic agent suitable for further development in cancer gene therapy.

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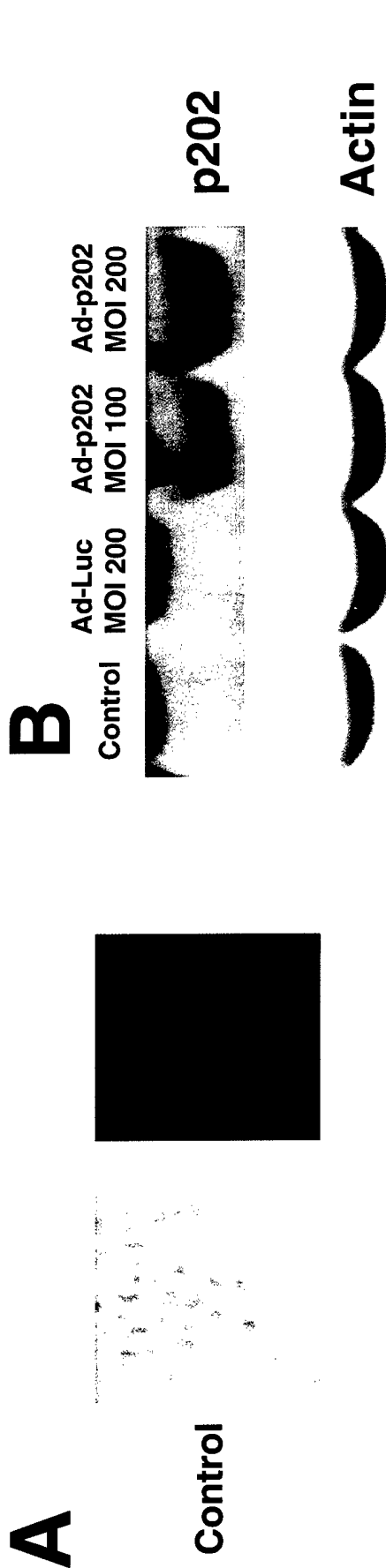


Fig. 1. Ad-p202 construction, p202 expression, and infection efficiency.

A. Ad-p202 was generated according to the protocol described previously .

The pAdTrack-CMV vector contains two independent CMV promoter-driven transcription units, one for GFP and one for p202 cDNA. Human breast cancer cells, MDA-MB-468, were infected by Ad-Luc or Ad-p202 at MOI of 200. 24-h post-infection, more than 90% cells were found to be GFP-positive as visualized by fluorescence microscopy (right panel), indicating the infection efficiency is more than 90%. Left panel: phase contrast microscopy. Control is the mock-infected cells. B. p202 protein is expressed in Ad-p202 infected cells. MDA-MB-468 cells infected with Ad-Luc or Ad-p202 for 72 h were analyzed for p202 protein expression by western blot. Control is the mock-infected cells. Actin protein was used as an equal loading control.



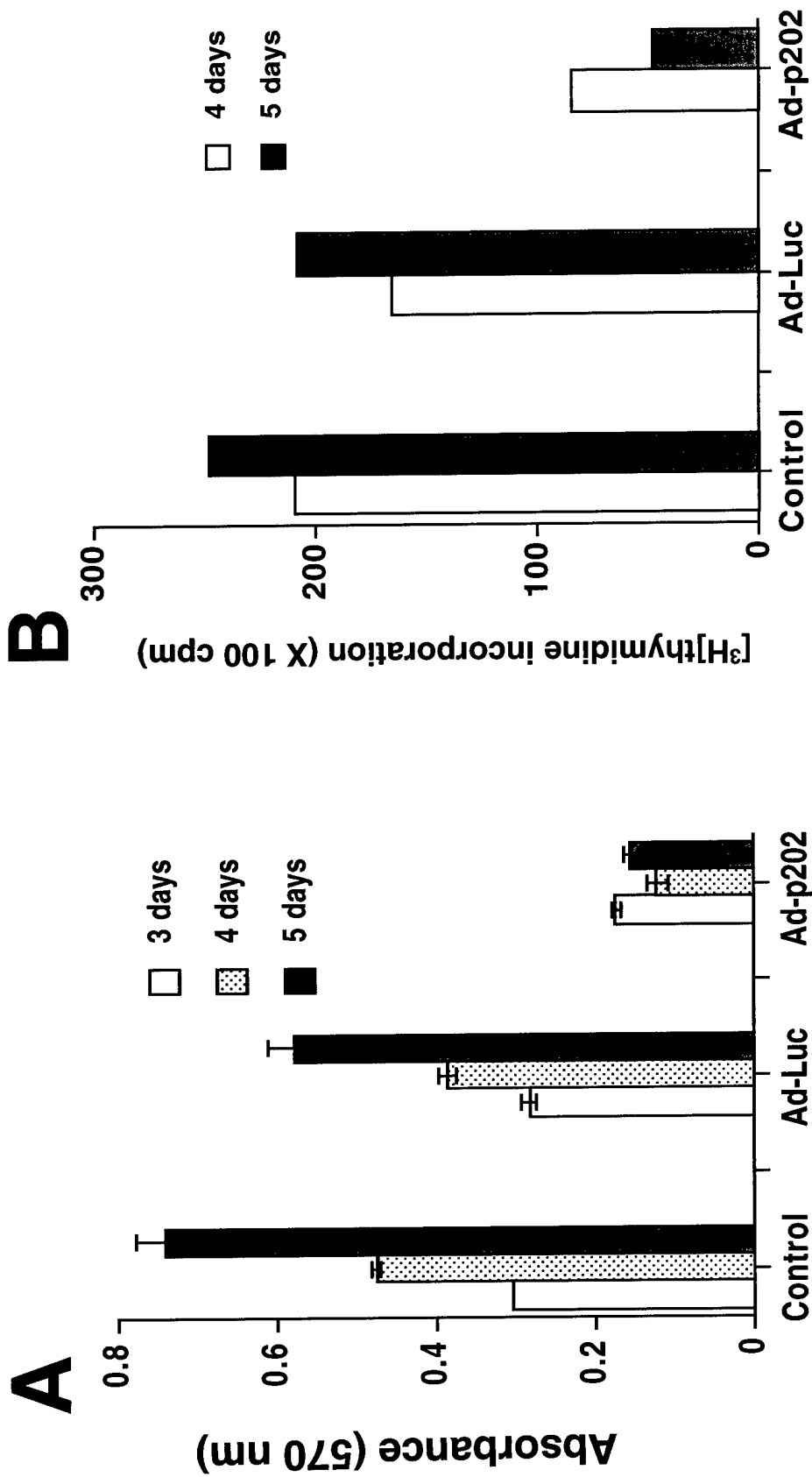


Fig. 2. Ad-p202 infection inhibits cell proliferation. MDA-MB-468 cells were infected with Ad-Luc or Ad-p202 at MOI of 200. Cell growth was monitored at the indicated post-infection time (3 - 5 days) by A, MTT assay, means  $\pm$  SD (n = 3), or B, [<sup>3</sup>H]-thymidine incorporation assay; data presented as means of quadruplicates.

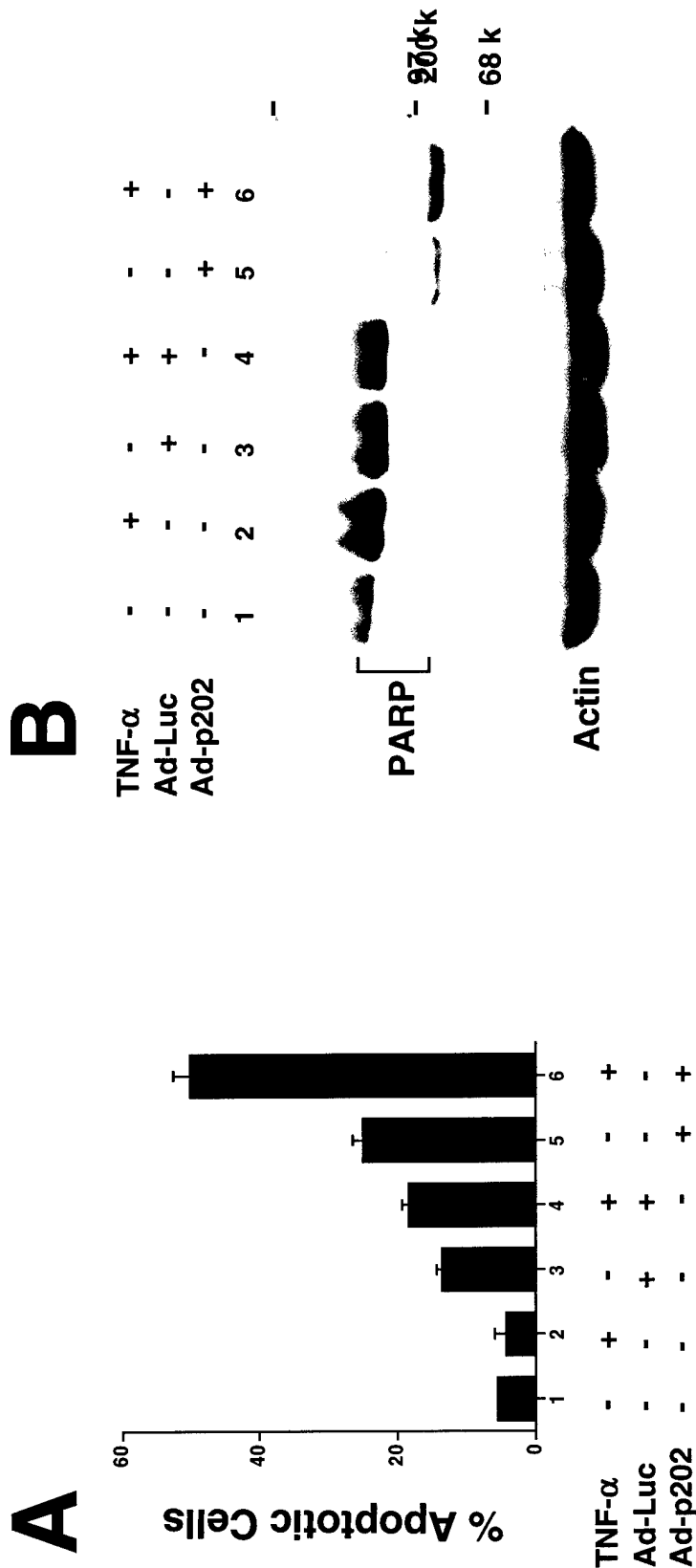


Fig. 3. Ad-p202 infection induces apoptosis and sensitizes cells to apoptosis induced by TNF- $\alpha$ . MDA-MB-468 cells were infected with Ad-Luc or Ad-p202 at MOI of 200. 24 h post-infection, TNF- $\alpha$  (50 ng/ml) was added to the medium and incubated for 48 h. A, 72 h post-infection, apoptosis was monitored by flow cytometry analysis (done in triplets, Bars: SD). B, PARP cleavage assay: 24 h post-infection, TNF- $\alpha$  (50 ng/ml) was added to the medium and incubated for 24 h. Treated cells were harvested 48 h post-infection.

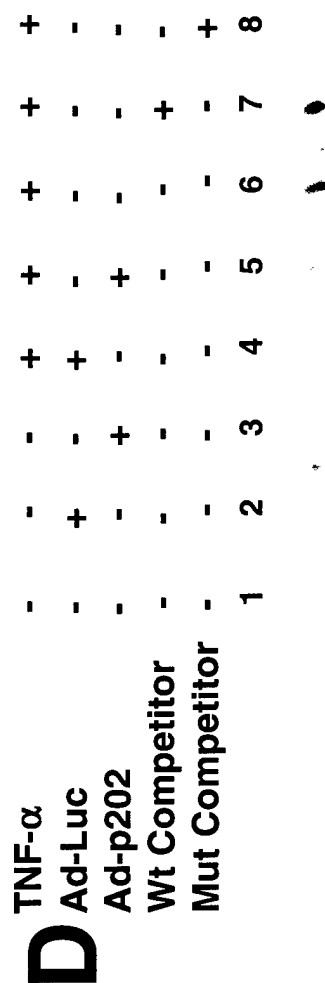
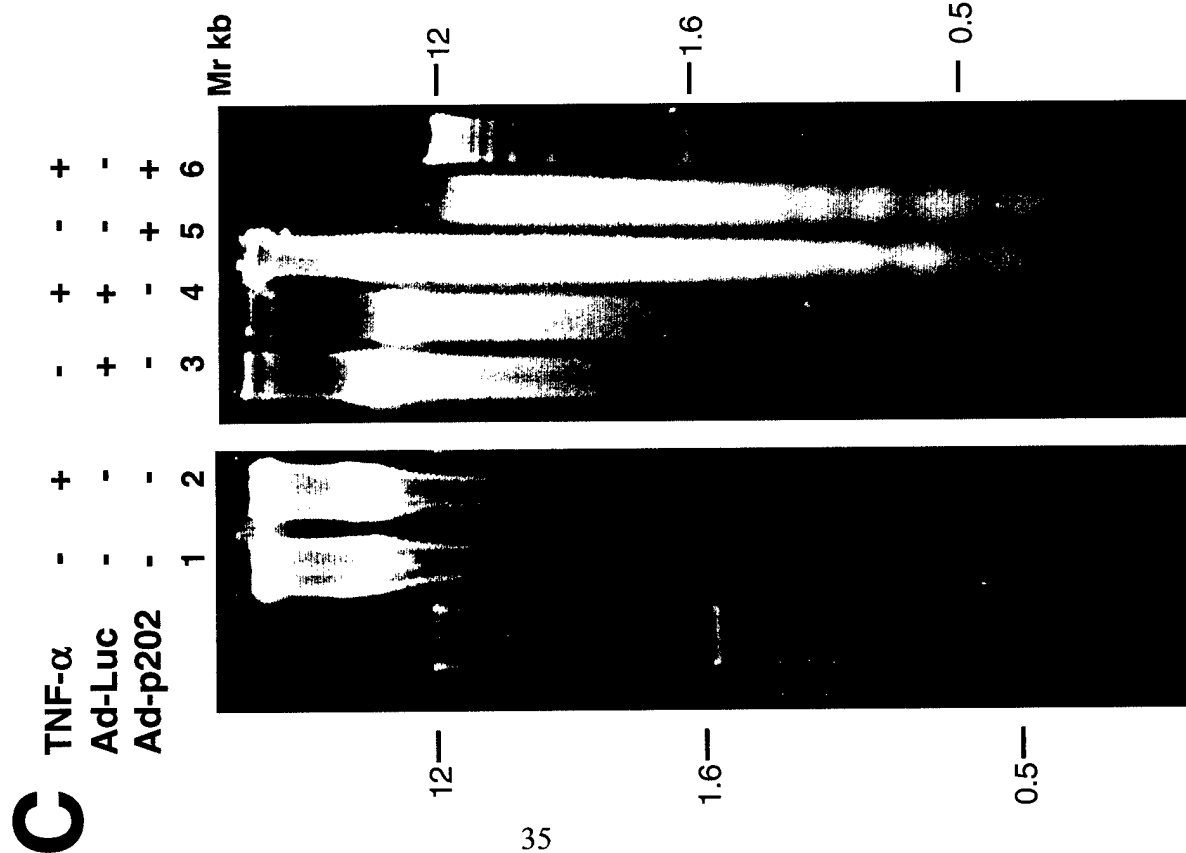


Figure 3 C, DNA fragmentation assay: 24 h post-infection, TNF- $\alpha$  (50 ng/ml) was added to the medium and incubated for 24 h. Treated cells were harvested 48 h post-infection. The PARP protein (116 kD) was cleaved into 85 kD product in the event of apoptosis. D. Ad-p202 infection inhibits the TNF- $\alpha$ -induced NF-kB DNA binding activity. MDA-MB-468 cells were infected with Ad-p202 or Ad-Luc in the presence or absence of TNF- $\alpha$  (50 ng/ml) 24 h post-infection for 30 minutes. The nuclear extracts were then isolated and incubated with a radioactive labeled oligonucleotide containing kB binding site. The excess cold wild type or mutant kB binding site were added to the incubation to demonstrate the specific NF-kB DNA binding activity. The NF-kB/DNA complex is indicated.

**A**

Z-VAD	-	-	-	+	+	-	-
Z-DEVD-fmk	-	-	-	-	-	+	+
Ad-Luc	-	+	-	+	-	+	-
Ad-p202	-	-	+	-	+	-	+
	1	2	3	4	5	6	7

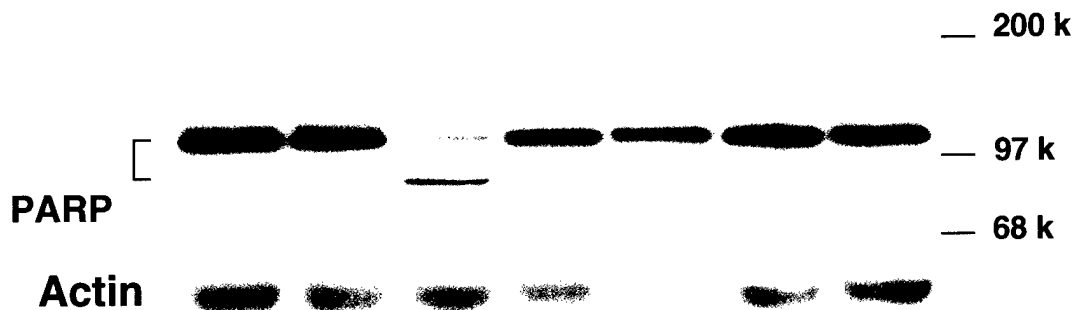
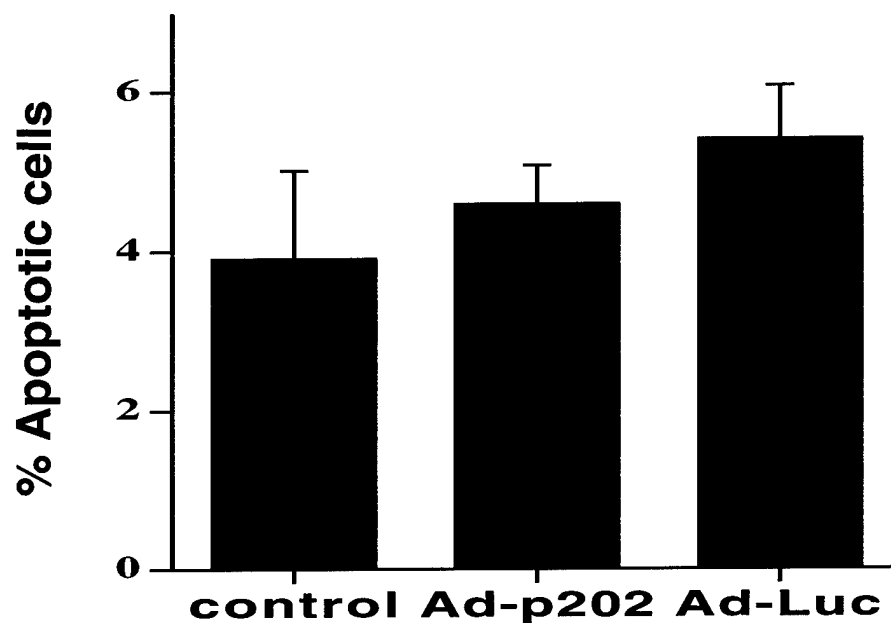
**B**

Fig. 4. The activation of caspases is critical for Ad-p202-mediated apoptosis. **A.** Z-VAD (100 mM) and Z-DEVD-fmk (80 mM) inhibit Ad-p202-mediated apoptosis in MDA-MB-468 cells. Western blot analysis of PARP cleavage and actin expression was performed 48-h post-infection. The intensity of PARP full-length and cleavage product bands were measured using NIH Image 1.62 software. **B.** Ad-p202 infection fails to induce apoptosis in MCF-7 cells. Flow cytometry analysis was used to measure apoptotic cell population in mock- (control), Ad-p202- (MOI of 2000), or Ad-Luc- (MOI of 2000) infected MCF-7 cells 72-h post-infection. The experiment was done in triplets. Bars: SD. p202 and actin protein expression was determined by western blot analysis.

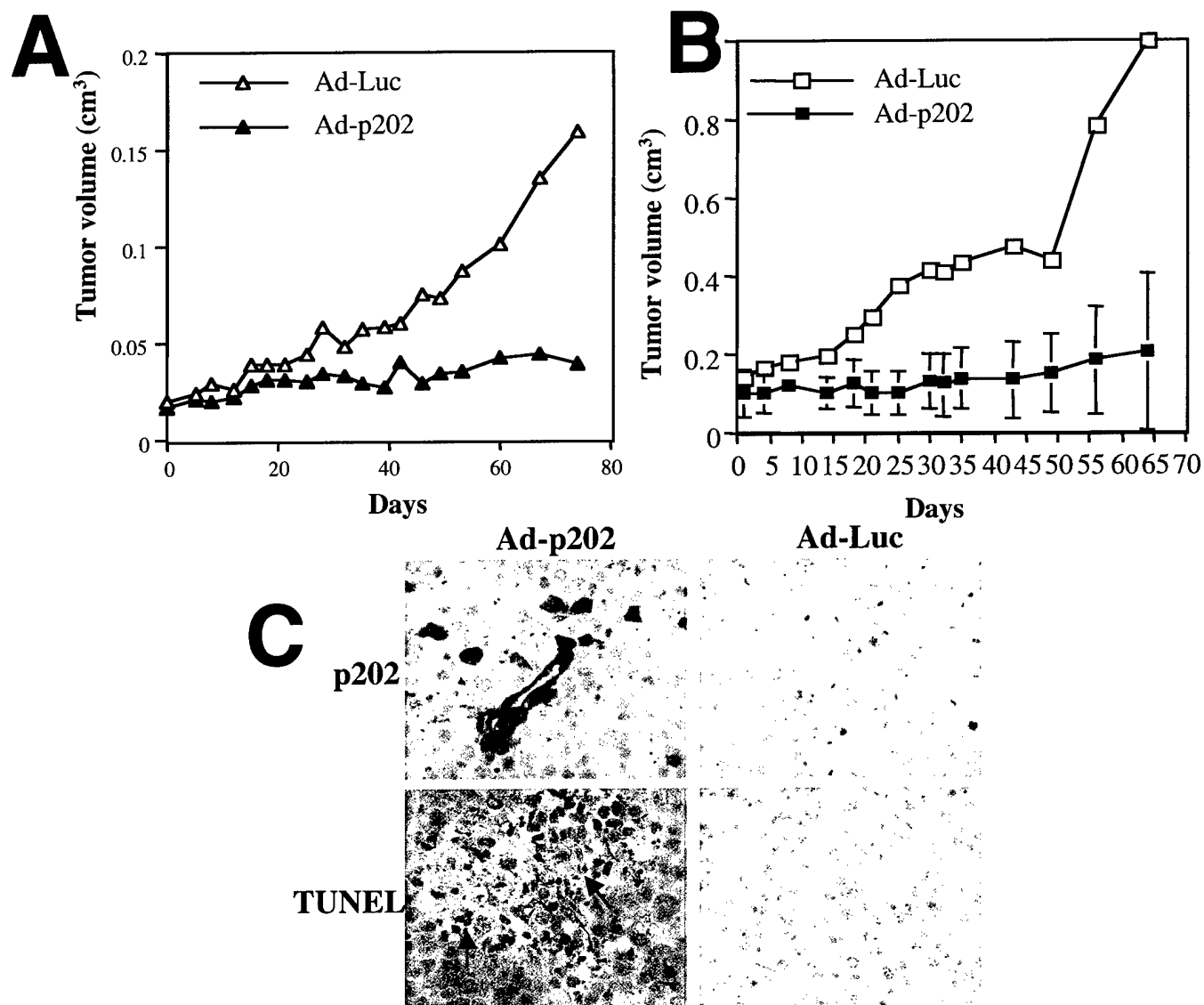
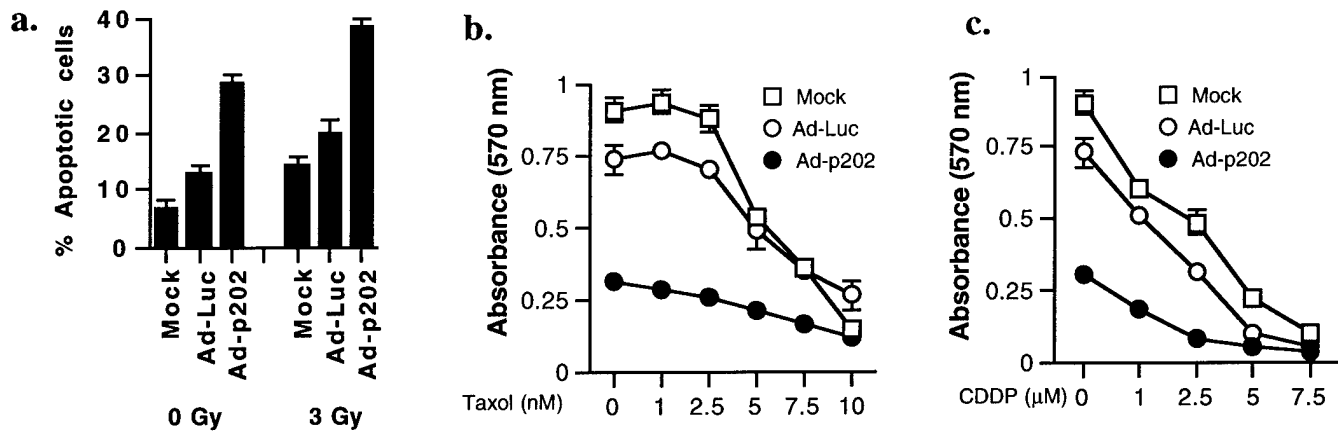
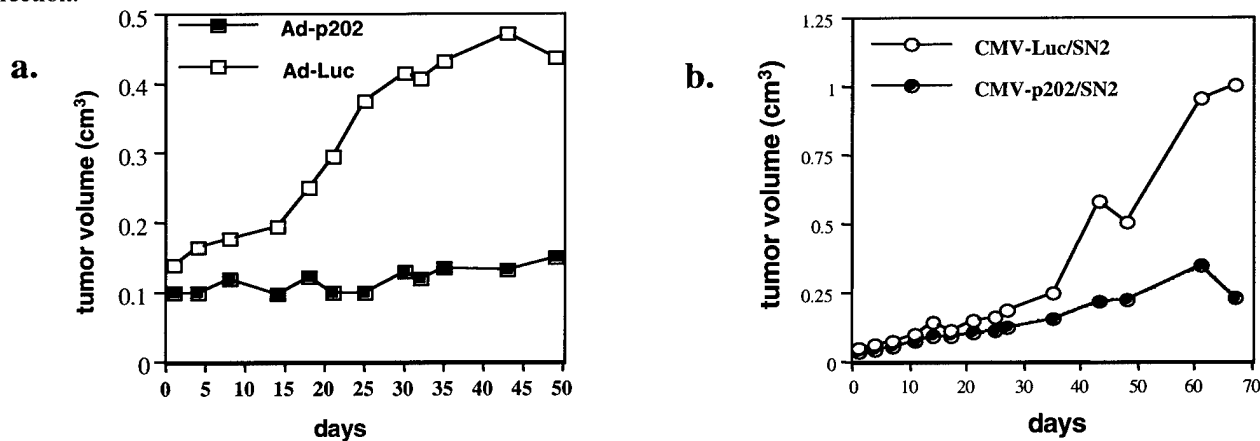


Fig. 5. Anti-tumor effect by systemic delivery of Ad-p202 on an orthotopic breast cancer xenograft model. **A.** Ad-p202-mediated anti-tumor effect on breast cancer xenografts by intra-tumor treatment. MDA-MB-468 cells ( $2 \times 10^6$  cells) were implanted in mammary fat pads (2 tumors per mouse) of each female nude mouse. Tumor-bearing mice were divided into two treatment groups: Ad-Luc (5 mice/10 tumors) and Ad-p202 (5 mice/10 tumors), at  $1 \times 10^9$  pfu per treatment via intra-tumor injection. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for seven weeks and once a week thereafter. Tumor volume was recorded at the time indicated. **B.** Ad-p202-mediated anti-tumor effect on breast cancer xenografts by systemic treatment. MDA-MB-468 cells ( $2 \times 10^6$  cells) were implanted in mammary fat pads (2 tumors per mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups: Ad-Luc (Luc) (2 mice/4 tumors) and Ad-p202 (p202) (3 mice/6 tumors), at  $5 \times 10^8$  pfu via tail vein injection. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for five weeks and once a week thereafter. Tumor volume was recorded at the time indicated. **C.** Apoptosis correlates with p202 expression in Ad-p202-treated breast tumors. Mice were sacrificed 24 h after the last treatment as described above. Tumors were then excised and fixed for the subsequent immunohistochemical analysis. p202 expression was analyzed by using antibody specific for p202 on tumor samples obtained from Ad-p202 or Ad-Luc treated mice. TUNEL assay was also performed to detect apoptotic cells in these tumors. The arrows indicate the representatives of apoptotic cells.



**Fig. 6. p202 expression enhances apoptosis induced by  $\gamma$ -irradiation, Taxol, or CDDP.** MDA-MB-468 cells were infected with Ad-p202, Ad-Luc at MOI of 200. Cells were treated with or without  $\gamma$ -irradiation (3 Gy) (a) or with increasing chemo-drugs, e.g., Taxol (b) and CDDP (c) at 24 h post-infection. Flow cytometry (a) and MTT assays (b and c) were performed at 72 h post-infection.



**Fig. 7. In vivo anti-tumor effect by a systemic delivery of Ad-p202 and CMV-p202/SN2.** a. MDA-MB-468 cells ( $1 \times 10^6$  cells) were implanted in each mammary fat pad of female nude mice. Tumor-bearing mice were divided into two treatment groups: group 1 (a), Ad-Luc (Luc) (2 mice/4 tumors) or Ad-p202 (p202) (3 mice/6 tumors), at  $5 \times 10^8$  pfu via tail vein injection; group 2 (b), CMV-Luc/SN2 (5 mice/10 tumors) or CMV-p202/SN2 (5 mice/10 tumors), at 20  $\mu$ g DNA/52  $\mu$ g SN2 in 200  $\mu$ l PBS via tail vein injection. Treatment started when tumor reached 5-cm in diameter with a treatment schedule of twice a week for five weeks and once a week thereafter. Tumor volume was recorded at the time indicated.

**Fig. 8. Immunohistochemical analysis of p202-treated tumors.** Mice were sacrificed 24 h after the last treatment as described in the legend of Fig. 7. Tumors were then excised and fixed for the subsequent analysis. Both p202 and VEGF expression were analyzed by using antibodies specific for p202 and human VEGF on tumor samples obtained from CMV-p202/SN2, CMV-Luc/SN2, Ad-p202, or Ad-Luc treated (via i.v. or i.t. injection) mice bearing either breast tumors derived from MDA-MB-468 cells or subcutaneous pancreatic tumors derived from PANC-1 cells. TUNEL assay was also performed to detect apoptotic cells in these tumors. The red color indicates positive for protein expression and for apoptosis. Blue color was used to stain for cell nuclei (Fig. 8 is on the next page)

**Fig. 8**

